



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 48/00, C12N 5/00, 15/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 99/21590</b> <b>(43) International Publication Date:</b> 6 May 1999 (06.05.99)
<b>(21) International Application Number:</b> PCT/US98/22668 <b>(22) International Filing Date:</b> 23 October 1998 (23.10.98)  <b>(30) Priority Data:</b> 60/063,629 27 October 1997 (27.10.97) US 9724906.4 26 November 1997 (26.11.97) GB  <b>(71) Applicant (for all designated States except US):</b> MERCK & CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> THOMAS, Kenneth, A., Jr. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). KENDALL, Richard, L. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). BETT, Andrew, J. [CA/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). HUCKLE, William, R. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).  <b>(74) Common Representative:</b> MERCK & CO., INC.; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).		<b>(81) Designated States:</b> AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GD, GE, HR, HU, ID, IL, IS, JP, KG, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TJ, TM, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> GENE THERAPY FOR STIMULATION OF ANGIOGENESIS  <b>(57) Abstract</b>  The present invention relates to methods of gene therapy to promote angiogenesis in the treatment of peripheral, cardiac and other pathological tissue ischemias utilizing a DNA molecule (SEQ ID NO:1) which encodes human VEGF145, set forth in SEQ ID NO:2.		

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

TITLE OF THE INVENTION  
GENE THERAPY FOR STIMULATION OF ANGIOGENESIS

CROSS-REFERENCE TO RELATED APPLICATIONS

5 Not applicable

STATEMENT REGARDING FEDERALLY-SPONSORED R&D  
Not applicable.

10 REFERENCE TO MICROFICHE APPENDIX  
Not applicable.

FIELD OF THE INVENTION

15 The present invention relates to methods of gene therapy to promote angiogenesis in the treatment of peripheral, cardiac and other pathological tissue ischemias utilizing a nucleotide sequence which encodes VEGF<sub>145</sub>.

BACKGROUND OF THE INVENTION

20 Vascular endothelial cells form a luminal non-thrombogenic monolayer throughout the vascular system. Mitogens promote embryonic vascular development, growth, repair and angiogenesis in these cells. Angiogenesis involves the proteolytic degradation of the basement membrane on which endothelial cells  
25 reside followed by the subsequent chemotactic migration and mitosis of these cells to support sustained growth of a new capillary shoot. One class of mitogens selective for vascular endothelial cells include vascular endothelial growth factor (referred to as VEGF or VEGF-A) and the homologues placenta growth factor (PlGF), VEGF-B, VEGF-C  
30 and VEGF-D.

Human VEGF exists as a glycosylated homodimer in one of five mature processed forms containing 206, 189, 165, 145 and 121 amino acids, the most prevalent being the 165 amino acid form.

35 U.S. Patent No. 5,240,848 discloses the nucleotide and amino acid sequence encoding the 189 amino acid form of human VEGF.

U.S. Patent No. 5,332,671 discloses the nucleotide and amino

acid sequence encoding the 165 amino acid form of human VEGF.

Charnock-Jones et al (1993, *Biol. Reproduction* 48: 1120-1128) and Sharkey et al (1993, *J. Reprod. Fertility* 99, 609-615) disclose a human VEGF<sub>145</sub> splice variant mRNA. Cheung et al. (1995, *Am. J. Obstet. Gynecol.* 173, 753-759) disclose an ovine VEGF<sub>145</sub> splice variant mRNA. These disclosures do not demonstrate the presence or activity of the protein product.

Poltorak et al. (1997, *J. Biol. Chem.* 272, 7151-7158) disclosed that recombinant human VEGF<sub>145</sub> has approximately one-sixth activity as a mitogen when compared to recombinant human VEGF<sub>165</sub>.

U.S. Patent No. 5,194,596 discloses the nucleotide and amino acid sequence encoding the 121 amino acid form of human VEGF.

The 206 amino acid and 189 amino acid forms of human VEGF each contain a highly basic 24-amino acid insert that promotes tight binding to heparin, and presumably, heparin proteoglycans on cellular surfaces and within extracellular matrices (Ferrara, et al., 1991, *J. Cell. Biochem.* 47: 211-218). The VEGF<sub>165</sub> form binds heparin to a lesser extent while VEGF<sub>121</sub> does not bind heparin.

Human PlGF is also a glycosylated homodimer which shares 46% homology with VEGF at the protein level. Differential splicing of human PlGF mRNA leads to either a 170 amino acid or 149 amino acid precursor, which are proteolytically processed to mature forms of 152 or 131 amino acids in length, respectively (Bayne and Thomas, EP Publication #0506477 [30 Sept 1992]; Maglione, et al., 1993, *Oncogene* 8: 925-931; Hauser and Weich, 1993, *Growth Factors* 9: 259-268).

VEGF-B has been isolated and characterized (Grimmond et al., 1996, *Genome Research* 6: 124-131; Olofsson et al., 1996, *Proc. Natl. Acad. Sci. USA* 93: 2576-2581). The full-length human cDNAs encode 188 and 207 amino acid residue precursors wherein the NH<sub>2</sub> terminal portions are proteolytically processed to mature forms 167 and 186 amino acid residues in length. Human VEGF-B expression was found predominantly in heart and skeletal muscle as a disulfide-linked homodimer. However, human VEGF-B may also form a heterodimer with VEGF (*id.* @ 2580).

VEGF-C has also been isolated and characterized (Joukov et

al., 1996, *EMBO J.* 15: 290-298; see also PCT International application WO 96/39515). A cDNA encoding VEGF-C was obtained from a human prostatic adenocarcinoma cell line. A 32 kDa precursor protein is proteolytically processed to generate the mature 23 kDa form, which  
5 binds the receptor tyrosine kinase, Flt-4.

VEGF-D was identified in an EST library, the full-length coding region was cloned and recognized to be most homologous to VEGF-C among the VEGF family amino acid sequences (Yamada, et al., 1997, *Genomics* 42:483-488). The human VEGF-D mRNA was shown  
10 to be expressed in lung and muscle.

VEGF and its homologues impart activity by binding to vascular endothelial cell plasma membrane-spanning tyrosine kinase receptors which then activate signal transduction and cellular signals. The Flt receptor family is a major tyrosine kinase receptor which binds  
15 VEGF with high affinity. At present the flt receptor family includes flt-1 (Shibuya, et al., 1990, *Oncogene* 5: 519-524), KDR/flk-1 (Terman, et al., 1991, *Oncogene* 6: 1677-1683; Terman, et al., 1992, *Biochem. Biophys. Res. Commun.* 187: 1579-1586), and flt-4 (Pajusola, et al., 1992, *Cancer Res.* 52: 5738-5743).

20 Vascular endothelial growth factor (VEGF) binds the high affinity membrane-spanning tyrosine kinase receptors KDR and Flt-1. Cell culture and gene knockout experiments indicate that each receptor contributes to different aspects of angiogenesis. KDR mediates the mitogenic function of VEGF whereas Flt-1 appears to modulate non-  
25 mitogenic functions perhaps including cellular adhesion and/or migration. Inhibiting KDR thus significantly diminishes the level of mitogenic VEGF activity.

Isner et al. (1996, *The Lancet* 348:370-374) disclose that administration of a DNA plasmid vector encoding recombinant human  
30 VEGF<sub>165</sub> to a human patient improved blood supply to an ischemic limb.

Despite recent advances in identifying genes encoding ligands and receptors involved in angiogenesis, there is no indication that gene therapy based on delivery and expression of VEGF<sub>145</sub> would promote the level of angiogenesis required to overcome peripheral or  
35 cardiac ischemias. The present invention addresses and meets this need.

## SUMMARY OF THE INVENTION

The present invention relates to methods of gene therapy for stimulating VEGF-induced angiogenesis associated with ischemic peripheral and/or cardiac muscle. Vascular endothelial growth factor acts as a mitogen to stimulate local angiogenesis from vascular endothelial cells so as to increase neovascularization, perfusion and performance of ischemic peripheral and/or cardiac muscle. A nucleic acid molecule encoding VEGF<sub>145</sub> or mutant versions thereof may be delivered either systemically or locally in a direct manner to target cells of the mammalian host by viral or non-viral based methods. A preferred mammalian host of the present invention is a human.

The present invention therefore relates to gene transfer of a nucleic acid molecule and concomitant *in vivo* expression of a soluble form of a mammalian VEGF<sub>145</sub> protein within a mammalian host. It is preferred that the form of VEGF used to practice the present invention be a mammalian splice variant related to human VEGF<sub>145</sub>. An especially preferred form for use in gene therapy application of the present invention is a DNA molecule encoding human VEGF<sub>145</sub>. It will be within the purview of the skilled artisan to generate one or more alternative forms of human VEGF<sub>145</sub>, a form which promotes angiogenesis on par with other forms of mammalian VEGF, and especially on par with other human VEGF forms, including but not limited to human VEGF<sub>189</sub>, human VEGF<sub>165</sub>, human VEGF<sub>121</sub>, human VEGF-B, human VEGF-C and human VEGF-D. Such a VEGF<sub>145</sub> gene therapy vehicle may be generated by recombinant DNA techniques known in the art using a DNA fragment encoding a partial or complete amino acid sequence of human VEGF<sub>145</sub>. Using recombinant DNA techniques, DNA molecules are constructed which encode at least a portion of human VEGF<sub>145</sub> receptor capable of stimulating angiogenesis. Standard recombinant DNA techniques are used such as those found in Maniatis, et al. (1982, Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) and which are exemplified within the confines of the specification.

In an especially preferred embodiment of the present invention, a DNA molecule comprising the nucleotide sequence as set forth in SEQ ID NO:1 is a template for constructing a gene therapy

vector. Such a gene therapy vector will express human VEGF<sub>145</sub> (SEQ ID NO:2) or a biologically active form of human VEGF<sub>145</sub> promotes angiogenesis subsequent to delivery to a mammalian host in order to combat cardiac or peripheral ischemia.

5 In another especially preferred embodiment of the present invention, a DNA molecule which encodes the human VEGF<sub>145</sub> protein as set forth in SEQ ID NO:2, or a biologically active form, is a template for constructing a gene therapy vector. Such a gene therapy vector will express human VEGF<sub>145</sub> (SEQ ID NO:2) or a biologically active form of  
10 human VEGF<sub>145</sub> and promote angiogenesis subsequent to delivery to a mammalian host in order to combat cardiac or peripheral ischemia.

Any VEGF<sub>145</sub> construct, including but not necessarily limited to a human VEGF<sub>145</sub> construct comprising the DNA sequence as set forth in SEQ ID NO:1, and biologically active form thereof, may be  
15 delivered to a mammalian host using a vector or other delivery vehicle. A DNA fragment encoding VEGF<sub>145</sub> or biologically active mutant versions thereof may be delivered either systemically or locally to target cells in the proximity of or within an ischemic tissue of a mammalian host by viral or non-viral based methods. Viral vector systems which  
20 may be utilized in the present invention include, but are not limited to, (a) adenovirus vectors; (b) retrovirus vectors; (c) adeno-associated virus vectors; (d) herpes simplex virus vectors; (e) SV 40 vectors; (f) polyoma virus vectors; (g) papilloma virus vectors; (h) picarnovirus vectors; and (i) vaccinia virus vectors. Non-viral methods of delivery include but are  
25 not necessarily limited to direct injection of naked DNA, such as any recombinant DNA plasmid expression vector described herein which comprises a DNA fragment encoding VEGF<sub>145</sub>. Additional non-viral vectors include but are not limited to DNA-lipid complexes, for example liposome-mediated or ligand/ poly-L-Lysine conjugates, such as  
30 asialoglyco-protein-mediated delivery systems.

A preferred viral vector of the present invention is a first, second or helper dependent adenovirus vector.

An especially preferred first generation recombinant Ad/VEGF<sub>145</sub> virus is AdVEGF<sub>145</sub>.

35 A preferred non-viral vector system of the present invention relates to use of a DNA plasmid expression vector, of which numerous

examples are known to the skilled artisan. As noted below, an expression vector is any polynucleotide having regulatory regions operably linked to a coding region such that, when in a host cell, the vector can direct the expression of the coding sequence. The expression  
5 vectors utilized to practice the present invention will comprise regulatory regions which promote expression within the target cell so as to impart a therapeutic effect on a particular ischemia within the mammalian host.

In addition to gene therapy related applications involving  
10 nucleic acid molecules encoding VEGF<sub>145</sub>, a VEGF<sub>145</sub> protein or biologically active fragment thereof may be utilized to treat various peripheral and/or cardiac ischemias in the mammalian host, preferably a human. Recombinant human VEGF<sub>145</sub> as exemplified within this specification may be delivered from slow release polymers or devices into  
15 ischemic tissue or systemically. Pharmaceutically useful compositions comprising VEGF<sub>145</sub> can be formulated according to known methods such as by the admixture of a pharmaceutically acceptable carrier. Examples of such carriers and methods of formulation can be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically  
20 acceptable composition suitable for effective administration, such compositions will contain a biologically effective amount of the VEGF<sub>145</sub> protein, preferably recombinant human VEGF<sub>145</sub> protein.

As used herein, "VEGF" or "VEGF-A" refers to vascular endothelial growth factor, which comprises proteins which are  
25 translational products of various splice variants, particularly VEGF<sub>121</sub>, VEGF<sub>145</sub>, VEGF<sub>165</sub> and VEGF<sub>189</sub>. A particular splice variant is referred to with the appropriate amino acid total of the mature form of the protein (e.g., VEGF<sub>121</sub>).

As used herein, "homologue of VEGF" refers to  
30 homodimers of VEGF-B, VEGF-C, VEGF-D and PlGF and any functional heterodimers formed between VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF, including but not limited to a VEGF-A/PlGF heterodimer.

As used herein, "VEGF-B" refers to vascular endothelial  
35 growth factor-B.

As used herein, "VEGF-C" refers to vascular endothelial



growth factor-C.

As used herein, "VEGF-D" refers to vascular endothelial growth factor-D.

As used herein, "KDR" or "FLK-1" refers to kinase insert  
5 domain-containing receptor or fetal liver kinase.

As used herein, "FLT-1" refers to fms-like tyrosine kinase receptor.

As used herein, "Ad" refers to adenovirus.

As used herein, "HUVECs" refers to human umbilical vein  
10 endothelial cells.

As used herein, the term "mammalian host" refers to any mammal, including a human being.

As used herein, the term "hVEGF<sub>145</sub>" refers to human VEGF<sub>145</sub>.

As used herein a "polynucleotide" is a nucleic acid of more  
15 than one nucleotide. A polynucleotide can be made up of multiple polynucleotide units that are referred to by description of the unit. For example, a polynucleotide can comprise within its bounds a polynucleotide(s) having a coding sequence(s), a polynucleotide(s) that is  
20 a regulatory region(s) and/or other polynucleotide units commonly used in the art.

As used herein, an "expression vector" is a polynucleotide having regulatory regions operably linked to a coding region such that, when in a host cell, the vector can direct the expression of the coding  
25 sequence. The use of expression vectors is well known in the art. Expression vectors can be used in a variety of host cells and, therefore, the regulatory regions are preferably chosen as appropriate for the particular host cell.

As used herein, a "biologically active fragment",  
30 "biologically active form", "biologically active equivalent" or "functional derivative" of a wild-type human VEGF<sub>145</sub> possesses a biological activity that is at least substantially equal to the biological activity of the wild type human VEGF<sub>145</sub>. The above-mentioned terms are intended to include "fragments", "mutants," or "variants," of the wild type human VEGF<sub>145</sub>  
35 protein which is not substantially similar to other known VEGF homologues. The term "fragment" is meant to refer to any polypeptide

subset of wild-type human VEGF<sub>145</sub> which is not substantially similar in structure to other known VEGF homologues. The term "mutant" is meant to refer to a molecule that may be substantially similar to the wild-type form but possesses distinguishing biological characteristics. Such altered characteristics include but are in no way limited to altered substrate binding, altered substrate affinity and altered sensitivity to chemical compounds affecting biological activity of the human VEGF<sub>145</sub> or human VEGF<sub>145</sub> functional derivative which may make the respective mutant attractive for the gene therapy applications disclosed within the confines of this specification. The term "variant" is meant to refer to a molecule substantially similar in structure and function to either the entire wild-type protein or to a fragment thereof.

It is an object of the present invention to provide systemic or localized delivery of VEGF<sub>145</sub> to a mammalian host, and preferably a human host, to stimulate angiogenesis for treatment of peripheral and cardiac ischemia.

It is also an object of the present invention to utilize a gene or gene fragment of human VEGF<sub>145</sub> in gene therapy methods to stimulate angiogenesis for treatment of peripheral and cardiac ischemia.

It is an object of the present invention to provide recombinant DNA vectors containing VEGF<sub>145</sub> constructs, preferably human VEGF<sub>145</sub> constructions, for use in gene therapy to stimulate angiogenesis for treatment of peripheral and cardiac ischemia.

25

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the cDNA sequence which encodes the full-length translation product precursor of mature human VEGF<sub>145</sub>. The TGA translation termination codon is shown and the sequence is grouped by codon.

Figure 2 shows the amino acid sequence of human VEGF<sub>145</sub> in single letter code, and as set forth in SEQ ID NO:2 in three letter code. The deduced amino acid sequence of the full-length translation product includes the predicted N-terminal 26 amino acid residue secretory leader sequence (underlined).

Figure 3 shows reversed-phase C4 Chromatography of

pooled VEGF<sub>145</sub>. The pooled VEGF<sub>145</sub>-containing sample from the Mono S column was loaded onto a C<sub>4</sub> reversed phase HPLC column equilibrated in 0.1% acetonitrile and eluted at flow rate of 200 µl/min with a 0-70% (v/v) gradient of acetonitrile monitoring absorbance at 215 nm.

Figure 4 shows a Western blot of hVEGF<sub>145</sub>. Fractions 15 and 16 from the C<sub>4</sub> HPLC chromatographic fractionation were analyzed by SDS/PAGE and Western blotting using an anti-VEGF antibody. The positions and masses in kDa of molecular mass markers are denoted on the right of the figure.

Figure 5 shows the purity of hVEGF<sub>145</sub>. Fraction 15 from the C<sub>4</sub> reversed phase HPLC column was analyzed by SDS/PAGE on a 4-20% gradient gel and silver stained. The positions and masses in kDa of molecular mass markers are denoted on the right of the figure.

Figure 6 shows VEGF<sub>145</sub> mitogenic activity. Purified human recombinant VEGF<sub>145</sub> (open circles) and VEGF<sub>165</sub> (filled circles) were assayed in parallel as a function of dose on human umbilical vein vascular endothelial cells in culture. Mitogenesis was monitored by incorporation of [<sup>3</sup>H]thymidine into DNA and expressed as percent maximum response. Each dose-response curve is the average of 3 separate determinations.

Figure 7 shows DNA plasmid expression vector, V1Jns.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods of gene therapy for stimulating VEGF-induced angiogenesis including but not limited to ischemic peripheral and/or cardiac tissue. Vascular endothelial growth factor acts as a mitogen to stimulate local angiogenesis from vascular endothelial cells so as to increase neovascularization, perfusion and performance of ischemic peripheral and/or cardiac muscle. A nucleic acid molecule encoding VEGF<sub>145</sub> or mutant versions thereof may be delivered either systemically or locally in a direct manner to target cells of the mammalian host by viral or non-viral based methods.

The present invention therefore relates to gene transfer of a nucleic acid molecule and concomitant *in vivo* expression of a soluble form of a mammalian VEGF<sub>145</sub> protein within a mammalian host. It is

preferred that the form of VEGF used to practice the present invention be a mammalian splice variant related to human VEGF<sub>145</sub>. An especially preferred form for use in gene therapy application of the present invention is a DNA molecule encoding human VEGF<sub>145</sub>.

5                   Therefore, the present invention relates in part to fully active human VEGF<sub>145</sub> to be delivered as a protein or gene therapeutic agent to promote angiogenesis. The cDNA fragment which encodes human VEGF<sub>145</sub> (as set forth in SEQ ID NO:1) was generated by PCR-based hybridization to human placenta total RNA, cDNA synthesis, and  
10 isolation of a positive clone for further characterization. The corresponding hVEGF<sub>145</sub> protein has been expressed from a baculovirus expression system in insect cells and chromatographically purified to homogeneity. Therefore, the present invention provides for expression of fully active recombinant VEGF<sub>145</sub> protein either in culture or by *in vivo*  
15 gene transfer delivery from plasmids or viral vectors to promote hVEGF<sub>145</sub> expression and to promote angiogenesis in and around ischemic tissue. Messenger RNA encoding a human alternatively spliced isoform of vascular endothelial growth factor (VEGF) that contains 145 amino acids in the mature processed form had been  
20 identified but the native protein was not demonstrated to exist so its effective translation and expression as a stably folded and active protein was not established. It is known that expression is not guaranteed by the presence of mRNA as shown in the case of several mRNAs encoding IGF II. A recombinant form of VEGF<sub>145</sub> was reported to be only 1/6th as  
25 active as VEGF<sub>165</sub> (Poltorak et al., 1997, *J. Biol. Chem.* 272: 7151-7158). Therefore, the disclosed gene therapy applications of the present invention are exemplified in part by showing that recombinant human VEGF<sub>145</sub> is equivalently active to human VEGF<sub>165</sub>. This data demonstrates, in stark contrast to the above-mentioned publication, that  
30 hVEGF<sub>145</sub> is a fully functional VEGF isoform. As a consequence of the full mitogenic activity of VEGF<sub>145</sub>, it is now established within the confines of this specification that VEGF<sub>145</sub> is an appropriate gene/protein for use as a therapeutic agent as indicated herein.

                  To this end, a particular embodiment of the present  
35 invention involves the use of a human recombinant form of VEGF<sub>145</sub> in gene therapy protocols. It will be within the purview of the skilled

artisan to generate additional forms of human VEGF<sub>145</sub> which are within the scope of the present invention. Any such biologically active alternative form will be structurally similar to VEGF<sub>145</sub> when compared to known VEGF homologues and will promote angiogenesis at a substantially similar level as with other such mammalian VEGF homologues, and especially at a substantially similar level as human VEGF homologues, including but not limited to human VEGF<sub>189</sub>, human VEGF<sub>165</sub>, human VEGF<sub>121</sub>, human VEGF-B, human VEGF-C and human VEGF-D. Such a VEGF<sub>145</sub> gene therapy vehicle may be generated by recombinant DNA techniques known in the art using a DNA fragment encoding a complete or partial amino acid sequence of human VEGF<sub>145</sub>. Again, any such partial or fragmented version of VEGF<sub>145</sub> will be substantially more similar to VEGF<sub>145</sub> as compared to other known forms of VEGF. Using recombinant DNA techniques, DNA molecules are constructed which encode at least a portion of human VEGF<sub>145</sub> receptor capable of stimulating angiogenesis. Standard recombinant DNA techniques are used such as those found in Maniatis, et al. (1982, Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

In a preferred embodiment of the present invention, a DNA fragment comprising the nucleotide sequence as set forth in SEQ ID NO:1 encoding human VEGF<sub>145</sub> is the template for constructing a gene therapy vector wherein expressed human VEGF<sub>145</sub> (SEQ ID NO:2) or a biologically active form promotes angiogenesis subsequent to delivery to a mammalian host in order to offer therapeutic treatment for cardiac or peripheral ischemia. Therefore, the present invention discloses methods of gene therapy utilizing a DNA molecule encoding VEGF<sub>145</sub> (preferably a DNA molecule encoding hVEGF<sub>145</sub>) and DNA molecules encoding biologically active fragments as noted herein, or various pharmaceutical applications of VEGF<sub>145</sub> protein (preferably hVEGF<sub>145</sub> protein) and biologically active fragments herein, to increase neovascularization, perfusion and performance of ischemic peripheral and cardiac muscle.

Briefly, a cDNA clone encoding human VEGF<sub>145</sub> was isolated by PCR-mediated screening of a human placental total RNA. The human placenta total RNA was used for the first strand cDNA. The

reaction was primed with random hexamers. Following the synthesis reaction aliquots (2 µl) were used as templates in polymerase chain reactions using Pfu DNA polymerase. The following primers derived from the rat cDNA sequence were used for the amplification of human VEGFs: forward primer,  
 5'ACGGGATCCAAATATGAACTTTCTGCTCTCTTG-3' (SEQ ID NO:3);  
 reverse primer, 5'-TGGAAGCTTTCACCGCCTTGGCTTGTC-3' (SEQ ID NO:4). Each primer contains a single nucleotide base change when compared to the isolated human DNA sequence. However the predicted protein sequence is identical to the human amino acid sequence (i.e., codon CTC → CTG for Pro as amino acid #5 of the signal sequence of VEGF<sub>145</sub> for the forward primer and codon CCA → CCG for Arg as amino acid # 143 of mature form of VEGF<sub>145</sub> for the reverse primer) as predicted from known sequence analysis and splice variant analysis of human VEGF homologues. PCR products were visualized by gel electrophoresis and a band corresponding to the expected size of VEGF<sub>145</sub> was detected only in the placental RNA reaction. The appropriate sized cDNA molecules were subcloned into a pCR-blunt plasmid, which was transfected into competent *E.coli* cells, grown, isolated and digested with EcoRI. Clones with the insert size expected for the human VEGF<sub>145</sub> gene fragment were confirmed by DNA sequence analysis.

A DNA fragment encoding human VEGF<sub>145</sub>, set forth as SEQ ID NO:1, is as follows:

```

25      ATG AAC TTT CTG CTC TCT TGG GTG CAT TGG
      AGC CTT GCC TTG CTG CTC TAC CTC CAC CAT
      GCC AAG TGG TCC CAG GCT GCA CCC ATG GCA
      GAA GGA GGA GGG CAG AAT CAT CAC GAA GTG
      GTG AAG TTC ATG GAT GTC TAT CAG CGC AGC
30      TAC TGC CAT CCA ATC GAG ACC CTG GTG GAC
      ATC TTC CAG GAG TAC CCT GAT GAG ATC GAG
      TAC ATC TTC AAG CCA TCC TGT GTG CCC CTG
      ATG CGA TGC GGG GGC TGC TGC AAT GAC GAG
      GGC CTG GAG TGT GTG CCC ACT GAG GAG TCC
35      AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA
      CCT CAC CAA GGC CAG CAC ATA GGA GAG ATG
  
```

AGC TTC CTA CAG CAC AAC AAA TGT GAA TGC  
AGA CCA AAG AAA GAT AGA GCA AGA CAA GAA  
AAA AAA TCA GTT CGA GGA AAG GGA AAG GGG  
CAA AAA CGA AAG CGC AAG AAA TCC CGG TAT  
5 AAG TCC TGG AGC GTG TGT GAC AAG CCA AGG  
CGG TGA (SEQ ID NO:1).

The human recombinant VEGF<sub>145</sub> protein expressed from  
SEQ ID NO:1 is set forth as SEQ ID NO:2 and is as follows:

MNFLLSWVHW SLALLLYLHH AKWSQAAPMA  
10 EGGGQNHHEV VKFMDVYQRS YCHPIETLVD IFQEYPDEIE  
YIFKPSCVPL MRCGGCCNDE GLECVPTES NITMQIMRIK  
PHQGQHIGEM SFLQHNKCEC RPKKDRARQE KKSVRGKGKG  
QKRKRKKSRY KSWSVCDKPR R (SEQ ID NO:2),

as shown in single letter code. The underlined portion represents the  
15 putative signal peptide for human VEGF<sub>145</sub>.

Expression vectors are defined herein as DNA sequences  
that are required for the transcription of cloned copies of genes and the  
translation of their mRNAs in an appropriate host. Such vectors can be  
used to express eukaryotic genes in a variety of hosts such as bacteria,  
20 bluegreen algae, fungal cells, yeast cells, plant cells, insect cells and  
animal cells.

Specifically designed vectors allow the shuttling of DNA  
between hosts such as bacteria-yeast or bacteria-animal or bacteria-  
insect cells. An appropriately constructed expression vector should  
25 contain: an origin of replication for autonomous replication in host cells,  
selectable markers, a limited number of useful restriction enzyme sites,  
a potential for high copy number, and active promoters. A promoter is  
defined as a DNA sequence that directs RNA polymerase to bind to DNA  
and initiate RNA synthesis. A strong promoter is one which causes  
30 mRNAs to be initiated at high frequency. Expression vectors may  
include, but are not limited to, cloning vectors, modified cloning vectors,  
specifically designed plasmids or viruses.

One embodiment of the present invention relates to a non-  
viral vector which is a recombinant plasmid vector comprising a  
35 nucleotide sequence encoding VEGF<sub>145</sub>. A preferred aspect of this  
embodiment is a recombinant plasmid vector which comprises a

nucleotide fragment which comprises human VEGF<sub>145</sub> as set forth in SEQ ID NO:1. It will be within the purview of the artisan of ordinary skill to pick and choose between available recombinant expression plasmids which express human VEGF<sub>145</sub> at therapeutically acceptable levels within the mammalian host.

In another especially preferred embodiment of the present invention, a DNA molecule which encodes the human VEGF<sub>145</sub> protein as set forth in SEQ ID NO:2, or a biologically active form, is a template for constructing a gene therapy vector. Such a gene therapy vector will express human VEGF<sub>145</sub> (SEQ ID NO:2) or a biologically active form of human VEGF<sub>145</sub> and promote angiogenesis subsequent to delivery to a mammalian host in order to combat cardiac or peripheral ischemia.

DNA encoding VEGF<sub>145</sub> or a biologically active fragment as defined herein may also be cloned into an expression vector for expression in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to bacteria, yeast, mammalian cells including but not limited to cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to drosophila, moth, mosquito and armyworm derived cell lines. The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection, Ad/polylysine DNA complexes, protoplast fusion, and electroporation. Cell lines derived from mammalian species which may be suitable and which are commercially available, include but are not limited to, CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171) and HEK 293 cells. Insect cell lines which may be suitable and are commercially available include but are not limited to 3M-S (ATCC CRL 8851) moth (ATCC CCL 80) mosquito (ATCC CCL 194 and 195; ATCC CRL 1660 and 1591) and armyworm (Sf9, ATCC CRL 1711) and Sf21 (Invitrogen).

Commercially available mammalian expression vectors which may be suitable for recombinant human VEGF<sub>145</sub> expression include but are not limited to, pcDNA3.1 (Invitrogen), pBlueBacHis2 or pBlue Bac 4 (Invitrogen), pLITMUS28, pLITMUS29, pLITMUS38 and



pLITMUS39 (New England Biolabs), pcDNAI, pcDNAIamp (Invitrogen), pcDNA3 (Invitrogen), pMC1neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593) pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460), and lZD35 (ATCC 37565).

The cloned human VEGF<sub>145</sub> cDNA obtained through the methods described above may be recombinantly expressed by molecular cloning into an expression vector containing a suitable promoter and other appropriate transcription regulatory elements, and transferred into prokaryotic or eukaryotic host cells to produce recombinant VEGF<sub>145</sub>. Techniques for such manipulations are fully described in Maniatis, et al.(id.), and are well known in the art. As a example, and not as a limitation, the VEGF<sub>145</sub> cDNA as set forth in SEQ ID NO:1 was expressed in a Baculovirus expression system for the purpose of obtaining purified preparations of hVEGF<sub>145</sub> and to test the ability of this protein to stimulate vascular endothelial cell mitogenesis necessary for angiogenesis. The hVEGF<sub>145</sub> gene fragment was isolated as a Bam HI/Hind III fragment and subsequently subcloned into the baculovirus expression vector pBlueBac4. Human VEGF<sub>145</sub> protein was expressed in Sf21 cells and concentrated through a heparin-Sepharose column. Human VEGF<sub>145</sub> fractions were identified loaded onto a Mono S HR5/5 column. Peak fractions were pooled, loaded onto and eluted from a C4 column. Human VEGF<sub>145</sub> was identified by Western blot and the purity was determined by separation on a 4-20% SDS/PAGE gel and then visualized by silver stain.

Additional expression vector and modifications thereof may be utilized which have been optimized for polynucleotide vaccinations. Essentially all extraneous DNA is removed, leaving the essential elements of transcriptional promoter, transcriptional terminator, bacterial origin of replication and antibiotic resistance gene. As noted throughout this specification, standard techniques of molecular biology for preparing and purifying DNA constructs enable the preparation of various DNA plasmid expression vectors. Numerous expression vectors which may be utilized to practice the gene therapy applications of the present invention are described in full within PCT International

Application WO97/31115, which is hereby incorporated by reference. For example, V1Jns is a DNA plasmid expression vector which comprises a CMV immediate-early (IE) promoter, bovine growth hormone (BGH) polyadenylation site, and a pUC backbone. It is also possible to replace  
5 the wild type signal sequence of VEGF<sub>145</sub> with a signal sequence from another protein, such as but not limited to tissue-specific plasminogen activator (tPA) gene, resulting in V1Jns-tPA. Additional DNA plasmid vectors described within WO97/31115 which may be used to practice the present invention in addition to V1Jns and V1Jns-tPA, includes but is  
10 not limited to V1Jneo. The nucleotide sequence of expression plasmid V1Jneo is set forth as SEQ ID NO:5. The expression plasmid V1Jns was constructed by introducing an Sfi I site into V1Jneo. A commercially available 13 base pair Sfi I linker (New England BioLabs) was added at the Kpn I site within the BGH sequence of the vector. V1Jneo was  
15 linearized with KpnI, gel purified, blunted by T4 DNA polymerase, and ligated to the blunt Sfi I linker. Clonal isolates were chosen by restriction mapping and verified by sequencing through the linker. The map of expression vector V1Jns is shown in Figure 7. To this end, a preferred, but in no way limiting plasmid expression vector which  
20 encodes human VEGF<sub>145</sub> is pV1JnsVEGF<sub>145</sub>, which is constructed as follows: A pCR-blunt clone described in Example Section 1 which comprises VEGF<sub>145</sub> (pCR-VEGF<sub>145</sub>-1) is digested with BamHI and EcoRI and ligated into BamHI/EcoRI digested pV1Jns, which will generate V1JnsVEGF<sub>145</sub>.

25 It is shown in Example Section 3 that purified recombinant human VEGF<sub>145</sub> stimulate proliferation of HUVEC monolayers in culture. It is known that expression of VEGF mitogenic receptors that mediate mitogenic responses to the growth factor is largely restricted to vascular endothelial cells. Human umbilical vein endothelial cells in  
30 culture proliferate in response to VEGF treatment and can be used as an assay system to quantify the effects VEGF isoforms. In the assay described in Example Section 3, quiescent HUVEC monolayers are stimulated to proliferate upon addition of VEGF. Purified baculovirus-expressed human VEGF<sub>145</sub> was fully efficacious as an endothelial cell  
35 mitogen when compared to baculovirus-expressed human VEGF<sub>165</sub> with equivalent half-maximal activities of 17-19 ng/ml for VEGF<sub>165</sub> and

VEGF<sub>145</sub>.

Therefore, any VEGF<sub>145</sub> construct, including but not necessarily limited to a human VEGF<sub>145</sub> construct comprising the DNA molecule as set forth in SEQ ID NO:1 or a DNA molecule which encodes the human VEGF<sub>145</sub> protein as set forth in SEQ ID NO:2, and biologically active forms thereof, may be delivered to the mammalian host using a vector or other delivery vehicle. As noted elsewhere in this specification, the preferred host of the present invention is a human host. In addition to a DNA plasmid expression vector as a DNA delivery vector for VEGF<sub>145</sub>-based gene therapy, other non-viral DNA delivery vehicles include but are not limited to DNA-lipid complexes, for example liposome-mediated or ligand/ poly-L-Lysine conjugates, such as asialoglyco-protein-mediated delivery systems (see for example: Felgner et al., 1994, *J. Biol. Chem.* 269:2550-2561; Derossi et al., 1995, *Restor. Neurol. Neuros.* 8:7-10; and Abcallah et al., 1995, *Biol. Cell* 85:1-7). It is preferred that local cells such as muscle cells be targeted for delivery and concomitant *in vivo* expression of the respective VEGF<sub>145</sub> protein to promote angiogenesis in and around the damaged tissue. A viral or non-viral recombinant gene therapy vehicle comprising a DNA fragment encoding VEGF<sub>145</sub> or mutant versions thereof may be delivered either systemically or locally to the target tissue and/or tissue adjacent to the ischemic region. However, other modes of administration of non-viral gene therapy vehicles are contemplated for this portion of the invention, including but not necessarily limited to subcutaneous, topical, oral, and intraperitoneal administration, all using forms well known to those of ordinary skill in the pharmaceutical arts.

Other DNA delivery vehicles include viral vectors such as adenoviruses, adeno-associated viruses, retroviral vectors (see, for example: Chu et al., 1994, *Gene Therapy* 1: 292-299; Couture et al., 1994, *Hum. Gene Therapy*. 5:, 667-277; and Eiverhand et al., 1995, *Gene Therapy* 2:336-343), or a combination system such as a recombinant chimeric adenoviral/retroviral vector system as described by Feng et al (1997, *Nature Biotechnology* 15(9): 866-870).

One such embodiment of the present invention is utilization of a first or second generation recombinant adenovirus (Ad) system for

systemic or local delivery of a DNA fragment encoding VEGF<sub>145</sub> or mutant versions thereof to the target cells of the mammalian host. A particularly useful first generation adenovirus system used to exemplify this portion of the present invention is described in Example Section 4. A first generation recombinant Ad/VEGF<sub>145</sub> is one preferred gene therapy vehicle for systemic or local delivery to ischemic tissue for the purpose of stimulating angiogenesis. An especially preferred recombinant Ad/VEGF<sub>145</sub> virus is AdVEGF<sub>145</sub>.

Another embodiment of the present invention is utilization of a helper-dependent recombinant adenovirus (Ad) system for systemic or local delivery of a DNA fragment encoding VEGF<sub>145</sub> or mutant versions thereof to the target cells of the mammalian. A particularly useful adenovirus system used to exemplify this portion of the present invention is described in Example Section 5 and is based on the system described by Parks et al. (1996, *Proc. Natl. Acad. Sci. (USA)* 93:13565-13570). A helper-dependent recombinant Ad/VEGF<sub>145</sub> is also a preferred gene therapy vehicle for systemic or local delivery of a VEGF<sub>145</sub>-encoding DNA fragment to ischemic tissue for the purpose of stimulating angiogenesis. An especially preferred helper-dependent recombinant Ad/VEGF<sub>145</sub> virus is AdHDVEGF<sub>145</sub>-1 or AdHDVEGF<sub>145</sub>-2.

The recombinant first, second or helper-dependent Ad/VEGF<sub>145</sub> viruses of the present invention, including but not limited to AdVEGF<sub>145</sub> (first generation), AdHDVEGF<sub>145</sub>-1 and AdHDVEGF<sub>145</sub>-2 (helper dependent viruses), are preferably administered to the host by direct injection into the area in and/or adjacent to ischemic tissue or quiescent tissue proximal to the area of ischemia, such as adipose or muscle tissue. It will of course be useful to transfect cells in the region of targeted adipose and muscle tissue. Transient expression of a VEGF<sub>145</sub> in these surrounding cells will result in a local extracellular increase in VEGF<sub>145</sub> and in turn will promote binding of recombinant VEGF to KDR to promote angiogenesis and in turn overcome the epoxic state associated with ischemia.

The recombinant first, second or helper-dependent Ad/VEGF<sub>145</sub> viruses of the present invention, including but not limited to AdVEGF<sub>145</sub>, AdHDVEGF<sub>145</sub>-1 and AdHDVEGF<sub>145</sub>-2, are also preferably delivered by i.v. injection. A recombinant adenovirus

delivered by i.v. injection will preferentially infect hepatocytes, where expression persists for approximately 3-4 weeks for a first generation vector and possibly longer for helper dependent vector subsequent to the initial infection. Suitable titers will depend on a number of factors, such as the particular vector chosen, the host, strength of promoter used and the severity of the disease being treated. The skilled artisan may alter the titer of virus administered to the patient, depending upon the method of delivery, size of the tumor and efficiency of expression from the recombinant virus. A dose in the range of  $10^6$ - $10^{11}$  plaque forming units (pfus) is preferred to treat most tissue ischemias with VEGF<sub>145</sub> therapy. The skilled artisan will also realize that the number of viral particles encoding the transgene, whether or not replication competent in a complementing host cell, are a relevant dosing unit. In most adenovirus constructs, there are 50 to 100-fold more DNA containing particles than pfus.

There are many embodiments of the instant invention which those skilled in the art can appreciate from the specification. To this end, different transcriptional promoters, terminators, carrier vectors or specific gene sequences may be used successfully. Optimal precision in achieving concentrations of expressed VEGF<sub>145</sub> within the range that yields optimal efficacy requires a regimen based on the kinetics of the proteins availability to appropriate membrane receptor kinases. This involves a consideration of the strength of expression from the VEGF<sub>145</sub> construct, distribution, equilibrium, and elimination of the protein.

The present invention provides methods of gene therapy which stimulate angiogenesis in and adjacent to ischemic tissue in a mammalian host, preferably a human host. It will be readily apparent to the skilled artisan that various forms of the nucleotide sequence(s) encoding human or any mutated version thereof may be utilized to alter the amino acid sequence of the expressed protein. The altered expressed protein may have an altered amino acid sequence, yet still bind to KDR and in turn promote angiogenesis. For example, it is preferred that expressed protein lack the entire signal sequence, that is that wild type proteolytic processing of the 26 amino acid signal sequence be complete. However, it is within the scope of the invention that the leader sequence

need not comprise the entire initial 26 amino acids of SEQ ID NO:2. In other words, the important point is that the final, mature product retain the ability to bind KDR and promote a mitogenic signal.

In an additional embodiment of the present invention a  
5 VEGF<sub>145</sub> protein or biologically active fragment thereof may be utilized to treat various peripheral and/or cardiac ischemias in the mammalian host. As an example but not forwarded as a limitation, recombinant human VEGF<sub>145</sub> as exemplified within this specification may be  
10 delivered from slow release polymers or devices into ischemic tissue or systemically. Pharmaceutically useful compositions comprising VEGF<sub>145</sub> can be formulated according to known methods such as by the admixture of a pharmaceutically acceptable carrier. Examples of such carriers and methods of formulation can be found in Remington's  
15 Pharmaceutical Sciences. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the VEGF<sub>145</sub> protein, preferably recombinant human VEGF<sub>145</sub> protein.

Therapeutic or diagnostic proteinaceous compositions of the invention are administered to an individual in amounts sufficient to  
20 treat or diagnose disorders. The effective amount can vary according to a variety of factors such as the individual's condition, weight, sex and age. Other factors include the mode of administration. The pharmaceutical compositions can be provided to the individual by a variety of routes such as subcutaneous, topical, oral and intramuscular.  
25 The term "chemical derivative" describes a molecule that contains additional chemical moieties which are not normally a part of VEGF<sub>145</sub>. Such moieties can improve the solubility, half-life, absorption, etc. of the protein. Examples of such moieties are described in a variety of texts, such as Remington's Pharmaceutical Sciences. Compounds identified  
30 according to the methods disclosed herein can be used alone at appropriate dosages. Alternatively, co-administration or sequential administration of other agents can be desirable.

The present invention also provides a means to obtain suitable topical, oral, systemic and parenteral proteinaceous  
35 pharmaceutical formulations for use in the methods of treatment of the present invention. The compositions containing compounds or

molecules identified according to this invention as the active ingredient can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compounds can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, they can also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts.

Advantageously, compounds of the present invention can be administered in a single daily dose, or the total daily dosage can be administered in divided doses of two, three or four times daily. Furthermore, compounds for the present invention can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

For combination treatment with more than one active agent, where the active agents are in separate dosage formulations, the active agents can be administered concurrently, or they each can be administered at separately staggered times.

The dosage regimen utilizing the compounds of the present invention is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal, hepatic and cardiovascular function of the patient; and the particular compound thereof employed. A physician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the drug required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentrations of VEGF<sub>145</sub> within the range that yields optimal efficacy requires a regimen based on the kinetics of the proteins availability to appropriate membrane receptor kinases. This involves a consideration of the distribution,

equilibrium, and elimination of the protein.

The following examples are provided to illustrate the present invention without, however, limiting the same hereto.

5

### EXAMPLE 1

#### ISOLATION OF A cDNA ENCODING HUMAN VEGF145

*cDNA synthesis and PCR amplification* - Five µg of human placenta total RNA (Clonetech Laboratories Inc. Cat # 64024-1) was used  
10 for the first strand cDNA synthesis in 20 µl reaction using SuperScript pre-amplification system (GIBCO BRL Life Technologies Cat. #18089-011) for first strand cDNA synthesis. The reaction was primed with random hexamers. Following the synthesis reaction aliquots (2 µl) were used as templates in polymerase chain reactions using Pfu DNA  
15 polymerase. The following primers were used for the amplification of human VEGFs: forward primer, 5'-ACGGGATCCAAATATGAACTTTCTGCTCTCTTG-3' (SEQ ID NO:3); reverse primer, 5'-TGGAAGCTTTCACCGCCTTGGCTTGTC-3' (SEQ ID NO:4). PCR reactions were performed as follows; to each PCR  
20 reaction tube add 5 µl of 10X PCR reaction buffer (10 X PCR reaction buffer is 100 mM KCl, 100 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 200 mM Tris-HCl, pH 8.75, 20 mM MgCl<sub>2</sub>, 2.0 mM dNTP, 1.0 % Triton X-100, 1 mg/ml bovine serum albumin [BSA]), 50 pmol of each primer and 2.5 units of Pfu DNA polymerase (Stratagene Cat. # 600153) in a total volume of 50 µl. The  
25 reaction was first denatured for 5 min at 95 °C, followed by 3 cycles of [2 min at 94 °C, 1.5 min at 54 °C, and 2 min at 72 °C] then 27 cycles of [1.5 min at 94 °C, 1 min at 68 °C, 1.5 min 72 °C] and finally 10 min at 72 °C. Two µl of the above reaction mixture was used as a template for a second round of PCR as follows; 5 min at 95 °C, then 25 cycles of [1.5 min at 94  
30 °C, 1 min at 68 °C, 1.5 min at 72 °C] followed by 10 min at 72 °C.

*VEGF145 cloning and confirmation* - Products from the second round of PCR were visualized by gel electrophoresis on a 1% agarose gel. DNA bands corresponding to the expected size of the human VEGF isoforms 121 and 165 were amplified using either HeLa  
35 cell or placental RNA. In addition, a band corresponding to the expected size of VEGF145 was detected only in the placental RNA reaction. The



appropriate size band was excised, purified and subcloned into pCR-blunt plasmid using the Zero blunt PCR cloning kit (Invitrogen Cat. # K2700-20). The plasmid was transfected into competent *E.coli* cells supplied with the kit and cDNAs generated from colonies selected for kanamycin resistance were digested with Eco RI then analyzed by gel electrophoresis. Clones with the insert size expected for a human VEGF<sub>145</sub> gene fragment were confirmed by DNA sequence analysis on an ABI 377 automatic sequencer. A VEGF<sub>145</sub> DNA molecule which encodes human VEGF<sub>145</sub> is shown in Figure 1 and is set forth as SEQ ID NO:1. The deduced amino acid sequence human VEGF<sub>145</sub> is shown in Figure 2 and set forth as SEQ ID NO:2.

## EXAMPLE 2

### EXPRESSION AND PURIFICATION OF RECOMBINANT HUMAN VEGF<sub>145</sub>

*Baculovirus expression of hVEGF<sub>145</sub>* - The hVEGF<sub>145</sub> gene fragment was isolated as a Bam HI/Hind III fragment and subsequently subcloned into the baculovirus expression vector pBlueBac4 (Invitrogen Cat. # V1995-20). The plasmid hVEGF<sub>145</sub>/pBB4 was transfected into Sf21 cells using Bac-N-Blue transfection kit (Invitrogen Cat. # K855-01). Recombinant virus was isolated by plaque purification and the virus stock was expanded by 3 rounds of infection at a multiplicity of infection (MOI) of 0.1 pfu/cell. Protein was produced by infecting Sf21 cells at an MOI of 5 pfu/cell at a cell density of  $1.5 \times 10^5$  cells/ml in HyQ serum-free medium (Hyclone Cat # SH30065.02). The infection was incubated at 27 °C for 72 hr with constant stirring and the medium was harvested by centrifugation (1000 x g for 10 min).

*Purification of recombinant VEGF<sub>145</sub>* - The concentration of hVEGF<sub>145</sub> was determined by a hVEGF ELISA (R & D Systems, Cat # DVE00) according to the manufacture's instruction using hVEGF<sub>165</sub> (Cat # 293-VE-010) supplied with the kit as a concentration standard. Typical hVEGF<sub>145</sub> expression levels were 200-400 µg/l of infected cells. Conditioned medium containing recombinant hVEGF<sub>145</sub> was directly loaded onto a 1 ml heparin-Sepharose column (Pharmacia Cat. #17-0406-01) equilibrated with phosphate buffer saline (PBS), pH 7.2. The column

was washed with PBS buffer containing 0.4 M NaCl, followed by a step elution with the same buffer containing 0.8 M NaCl. Fractions were analyzed for VEGF by SDS/PAGE followed by Western blotting using a polyclonal antibody (MSD88) raised against recombinant human VEGF165. Peak fractions containing hVEGF145 were pooled, diluted 16-fold with H<sub>2</sub>O, and then loaded onto a Mono S HR5/5 column (Pharmacia Cat. # 17-0547-01). The column was eluted with a linear gradient (0-100%) from 0.5X PBS, pH 7.2 to 0.8 M NaCl in PBS, pH 7.2 at a flow rate of 0.5 ml/min. Peak fractions were pooled and loaded onto a 4.6 mm x 5 cm C4 column (Vydac Cat# 214TP5405) then eluted with a 0-100% linear gradient (1%/min) from 0.1% trifluoroacetic acid to 70% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 200 µl/min (Figure 3). VEGF145 was identified by Western blot (Figure 4) and the purity was determined by separation on a 4-20% SDS/PAGE gel then visualized by silver stain (Figure 5). Closely spaced bands most likely represent microheterogenous forms often times associated with baculovirus expression systems.

### EXAMPLE 3

#### BIOLOGICAL ACTIVITY OF HUMAN VEGF<sub>145</sub>

##### *Human Umbilical Vein Endothelial Cell Mitogenesis*

**Assay-** Expression of VEGF mitogenic receptors that mediate mitogenic responses to the growth factor is largely restricted to vascular endothelial cells. Human umbilical vein endothelial cells (HUVECs) in culture proliferate in response to VEGF treatment and can be used as an assay system to quantify the effects VEGF isoforms. In the assay described in this Example Section, quiescent HUVEC monolayers are stimulated to proliferate with VEGF. The mitogenic response as a function of VEGF is determined by measuring the incorporation of [<sup>3</sup>H]thymidine into cellular DNA.

**Methods -** HUVECs frozen as primary culture isolates are obtained from Clonetics Corp. Cells are maintained in Endothelial Growth Medium (EGM; Clonetics) and are used for mitogenic assays at passages 3-7. Monolayers maintained in EGM are harvested by trypsinization and plated at a density of 4000 cells per 100 µl Assay

Medium per well in 96-well plates (NUNC/NON 96-well polystyrene tissue culture plates [NUNC #167008]). Cells are growth-arrested for 24 hours at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. After the 24-hour quiescent period, 10 µl/well of Assay Medium (Dulbecco's modification of Eagle's medium containing 1 mg/ml glucose [low-glucose DMEM; Mediatech] plus 10% (v/v) fetal bovine serum [Clonetics]) containing 10X VEGF solutions are added over a concentration range spanning the mitogenic dose/response curve. Cells are then incubated at 37 °C/5% CO<sub>2</sub>. Solutions of purified human VEGF165 (500 ng/ml; R&D Systems, expressed in Sf21 cells) and purified human VEGF145 were prepared in Assay Medium. Concentrations of VEGF isoforms were determined by using an enzyme-linked immunosorbent assay (R&D systems). After 24 hours in the presence of growth factors, 10X [<sup>3</sup>H]Thymidine (10 µl/well) is added. 10X [<sup>3</sup>H]Thymidine is [Methyl-<sup>3</sup>H]Thymidine (20 Ci/mmol; Dupont-NEN), diluted to 80 µCi/ml in low-glucose DMEM. Three days after addition of [<sup>3</sup>H]thymidine, medium was removed by aspiration, and cells are washed twice with Cell Wash Medium (400 µl/well followed by 200 µl/well). Cell Wash medium was Hank's balanced salt solution (Mediatech) containing 1 mg/ml bovine serum albumin (Boehringer-Mannheim). The washed, adherent cells are then solubilized by addition of Cell Lysis Solution (100 µl/well) and warming to 37 °C for 30 minutes. Cell Lysis Solution is 1 N NaOH, 2% (w/v) Na<sub>2</sub>CO<sub>3</sub>. Cell lysates are transferred to 7 ml glass scintillation vials containing 150 µl of water. Scintillation cocktail (5 ml/vial) is added, and cell-associated radioactivity is determined by liquid scintillation spectroscopy.

*Results* - Purified baculovirus-expressed human VEGF145 was fully efficacious as an endothelial cell mitogen when compared to baculovirus-expressed human VEGF165 (Figure 6) with equivalent half-maximal activities of 17-19 ng/ml for VEGF165 and VEGF145.

#### EXAMPLE 4

#### CONSTRUCTION OF THE FIRST GENERATION

#### ADENOVIRUS VECTOR ADVEGF145

35

Several systems have been developed for the construction of

first generation adenovirus vectors and have been recently reviewed by Graham and Prevec (1995, *Mol. Biotech.* 3: 207-220) and Hitt et al. (1995, Techniques for human adenovirus vector construction and characterization, In *Methods in Molecular Genetics, Volume 7.*

5 *Molecular Virology Techniques Part B*, ed. Kenneth W. Adolph, Academic Press, Inc. Orlando, Florida). All of these systems involve cloning the transgene of interest (coding region flanked by appropriate regulatory sequences) into a shuttle plasmid in which it is flanked by Ad sequences homologous to the region of the viral genome into which the  
10 transgene will be introduced. The transgene is then rescued into virus by either direct ligation *in vitro* followed by transfection into 293 cells, homologous recombination in bacteria followed by transfection into 293 cells (Chartier et al., 1996, *Journal of Virology* 70: 4805-4810), or by  
15 *in vivo* homologous recombination following transfection into 293 cells.

E1 shuttle plasmids have been developed for the rescue of inserts into the E1 region. These plasmids contain the left 16% of the Ad genome with a deletion of E1 sequences and cloning sites into which the transgene is introduced. If convenient restriction sites are available in the vector backbone, direct ligation of the shuttle plasmid to purified  
20 viral DNA can be performed *in vitro* followed by transfection into 293 cells to generate infectious virus. This method although efficient can require extensive screening if the viral DNA is not completely restricted and in many cases is not practical due to the lack of unique correctly positioned restriction sites. For these reasons many protocols rely on *in*  
25 *vivo* homologous recombination to generate infectious virus.

To construct a virus by homologous recombination in 293 cells the E1 shuttle plasmid can be transfected into 293 cells with purified viral DNA that has been restricted in the left end or with viral DNA contained in a second plasmid (an Ad genome plasmid). As with  
30 direct ligation the use of purified viral DNA sometimes requires extensive screening to obtain the desired vector because of the regeneration of parental virus and for this reason plasmid systems are more desirable. A number of Ad genome plasmid systems have been developed for rescuing inserts into E1 (McGrory et al., 1988, *Virology* 163:  
35 614-6170) or E3 (Ghosh-Choudhury, et al., 1986, *Gene* 50: 161-171; Mittal, et al., 1993, *Virus Res.* 28: 67-90) or both (Bett et al., 1994, *Proc. Natl.*

*Acad. Sci. USA* 91: 8802-8806) regions.

To construct a virus by homologous recombination in *E. coli* a segment of the E1 shuttle plasmid containing the transgene flanked by adenoviral sequences is gel purified and used to transform *E. coli* along  
5 with an Ad genome plasmid which has been linearized in the region in which the transgene is to be rescued. Homologous recombination between the two DNA's results in a repaired plasmid which can then be selected, grown up and purified from the bacteria and used to transfect 293 cells to generate virus.

10 To construct the first generation vector expressing VEGF145 the system involving homologous recombination in *E. coli* was used (Chartier et al., 1996, *Journal of Virology*, 70: 4805-4810). The steps involved in the construction are outlined below. The coding sequences for VEGF145 were obtained from the pCR-blunt clone described above by  
15 digestion with BamHI and EcoRI and cloned into the E1 shuttle plasmid pHCMVI1BGHpA-2, generating pHCMVI1VEGF145. To remove an undesirable PacI restriction site pHCMVI1VEGF145 was digested with PacI, treated with T4 DNA polymerase and religated, generating pHCMVI1VEGF145P-  
20 pHCMVI1VEGF145P- was then digested with SspI and Bst1107I and the fragment containing the transgene flanked by Ad sequences was gel purified. The purified fragment was then used to transform *E. coli* strain BJ5183 along with Ad genome plasmid pHVAd1 that was linearized in the E1 region by ClaI digestion. PHVAd1 contains the entire Ad genome with a deletion of E3 sequences from Ad bp 28133 to  
25 bp308180 and has the viral ITR's separated by plasmid sequences which contain the Ampicillin resistance gene and bacterial origin of replication. Homologous recombination between the purified shuttle plasmid fragment and linearized pHVAD1 generated a repaired plasmid designated pHVAdVEGF145P-. Bacterial transformants  
30 carrying pHVAdVEGF145P- were isolated and the plasmid DNA extracted and used to transform *E. coli* strain HB101 in which the plasmid grows more efficiently. pHVAdVEGF145P- plasmid DNA extracted and purified from HB101 cultures was digested with PacI to liberate the viral ITR's from plasmid DNA sequences and used to  
35 transfect 293 cells. The virus AdVEGF145 was obtained from this transfection.

EXAMPLE 5  
CONSTRUCTION OF THE HELPER DEPENDENT ADENOVIRUS  
VECTORS ADHDVEGF145-1 AND ADHDVEGF145-2

5           Helper-dependent Ad vectors are deleted of all viral coding sequences and contain only the *cis* acting viral sequences needed for DNA replication (the ITR's 1-103 bp located at each end of the genome) and genome encapsidation (packaging signals 194-358 bp). The helper-dependent vector carries the transgene and "stuffer" DNA (noncoding  
10 DNA) required to generate a vector that is efficiently packaged. For efficient packaging the vector genome should not be less than 75% (approximately 28 Kb) and the upper limit not more than 105% (approximately 38 Kb) of the wt Ad genome size of 36 Kb. All other viral proteins are provided in *trans* from a helper virus.

15           The helper virus AdLC8cLUC is an E1-deleted first generation vector which contains *lox* P sites flanking its packaging signals. When 293 cells expressing the *cre*-recombinase are coinfectd with the helper virus and dependent vector, the packaging signals are excised from the helper virus preventing it from being encapsidated,  
20 while allowing its genome to provide functions in *trans* to the dependent vector. Five to six serial passages are needed to increase the titer of the helper-dependent vector prior to a large-scale amplification from which vector is purified on cesium chloride gradients.

          The steps involved in the construction of the helper-  
25 dependent Ad vectors expressing VEGF145 are outlined below. The methods for the construction of helper-dependent Ad vectors are described in Parks et al (1996, *Proc. Natl. Acad. Sci.* 93: 13565-13570). The coding sequences for VEGF145 were obtained from the pCR-blunt clone described above by digestion with BamHI and EcoRI and cloning  
30 into the plasmid expression vector pV1Jns (described above and in PCT International Application W097/31115), generating pV1JnsVEGF145. The transgene cassette was then removed from pV1JnsVEGF145 by digestion with SfiI and MscI, treated with T4 DNA polymerase to generate blunt ends and cloned into the HindII site in helper dependent  
35 shuttle plasmid pABSHD-3, generating pSHDVEGF145-1 and pSHDVEGF145-2. Helper-dependent shuttle plasmid pABSHD-3

contains a multiple cloning region adjacent to a kanamycin resistance gene that allows for the selection of the desired recombinant plasmid after cloning into the ampicillin resistance gene containing helper-dependent backbone plasmid pSTK120. The transgene/Kan cassette was  
5 removed from pSHDVEGF145-2 by FseI digestion and cloned into pSTK120 generating pSTKVEGF145Kan-1 and pSTKVEGF145Kan-2. Finally, the kanamycin resistance gene was removed from both pSTKVEGF145Kan-1 and pSTKVEGF145Kan-2 by digestion with AscI followed by ligation, generating pSTKVEGF145-1 and pSTKVEGF145-2  
10 respectively. pSTKVEGF145-1 and pSTKVEGF145-2 were then digested with PmeI to release the viral ITR's from plasmid sequences and transfected into 293 cells, which were infected with helper virus AdLC8cLUC 24 hours later. When the cells were completely lysed the medium was collected and used to infect 293cre4 cells. Five serial  
15 passages in 293cre4 cells were required to increase the titer of the helper dependent vectors prior to large-scale vector purification.

## WHAT IS CLAIMED:

1. A method of stimulating angiogenesis in a mammalian host which comprises delivering a DNA vector to said mammalian host, said DNA vector expressing VEGF<sub>145</sub> or a biologically active fragment thereof.
2. The method of claim 1 wherein said mammalian host is a human.
3. The method of claim 2 wherein said DNA vector is a recombinant adenovirus.
4. The method of claim 2 wherein said DNA vector is a recombinant DNA plasmid vector.
5. The method of claim 3 wherein said recombinant adenovirus is delivered by infection into cells within or adjacent to a tissue ischemia.
6. A method of stimulating angiogenesis in a mammalian host which comprises delivering a DNA vector to said mammalian host, said DNA vector expressing human VEGF<sub>145</sub> as set forth in SEQ ID NO:1, or a biologically active fragment thereof.
7. The method of claim 6 wherein said mammalian host is a human.
8. The method of claim 7 wherein said DNA vector is a recombinant adenovirus.
9. The method of claim 7 wherein said DNA vector is a recombinant DNA plasmid vector.
10. The method of claim 8 wherein said recombinant DNA plasmid vector is delivered by injection into cells within or adjacent



to a tissue ischemia.

11. The method of claim 8 wherein said recombinant adenovirus is delivered by infection into cells within or adjacent to an  
5 ischemic peripheral or cardiac tissue.

12. The method of claim 11 wherein said recombinant adenovirus is AdVEGF145.

13. The method of claim 11 wherein said recombinant adenovirus is AdHDVEGF145-1.

14. The method of claim 11 wherein said recombinant adenovirus is AdHDVEGF145-2.

15. The method of claim 9 wherein said recombinant DNA plasmid vector is delivered by injection into cells within or adjacent to a tissue ischemia.

16. The method of claim 9 wherein said recombinant adenovirus is delivered by infection into cells within or adjacent to an ischemic peripheral or cardiac tissue.

17. The method of claim 16 wherein said recombinant DNA plasmid vector is pV1JnsVEGF145.

18. A recombinant virus comprising a DNA fragment encoding humanVEGF145 containing at least one regulatory sequence which controls expression of said DNA fragment within a mammalian  
30 host.

19. A recombinant virus of claim 18 which is a recombinant adenovirus.

20. A recombinant adenovirus of claim 19 wherein said DNA fragment encodes a human VEGF145 as set forth in SEQ ID NO:2.

21. A recombinant adenovirus of claim 20 selected from the group consisting of AdVEGF-145, AdVEGF145-1 and AdVEGF145-2.

1/6

ATG AAC TTT CTG CTC TCT TGG GTG CAT TGG  
AGC CTT GCC TTG CTG CTC TAC CTC CAC CAT  
GCC AAG TGG TCC CAG GCT GCA CCC ATG GCA  
GAA GGA GGA GGG CAG AAT CAT CAC GAA GTG  
GTG AAG TTC ATG GAT GTC TAT CAG CGC AGC  
TAC TGC CAT CCA ATC GAG ACC CTG GTG GAC  
ATC TTC CAG GAG TAC CCT GAT GAG ATC GAG  
TAC ATC TTC AAG CCA TCC TGT GTG CCC CTG  
ATG CGA TGC GGG GGC TGC TGC AAT GAC GAG  
GGC CTG GAG TGT GTG CCC ACT GAG GAG TCC  
AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA  
CCT CAC CAA GGC CAG CAC ATA GGA GAG ATG  
AGC TTC CTA CAG CAC AAC AAA TGT GAA TGC  
AGA CCA AAG AAA GAT AGA GCA AGA CAA GAA  
AAA AAA TCA GTT CGA GGA AAG GGA AAG GGG  
CAA AAA CGA AAG CGC AAG AAA TCC CGG TAT  
AAG TCC ATG AGC GTG TGT GAC AAG CCA AGG  
CGG TGA (SEQ ID NO:1)

**FIG.1**

MNELLSWVHW SLALLLYLHH AKWSOAAPMA EGGGQNHHEV  
VKFMDVYQRS YCHPIETLVD IFQEYPDEIE YIFKPSCVPL  
MRCGGCCNDE GLECVPTES NITMQIMRIK PHQGQHIGEM  
SFLQHNKCEC RPKKDRARQE KKSVRGKGKG QKRKRKKSRY  
KWSVCDKPR R (SEQ ID NO:2)

**FIG.2****SUBSTITUTE SHEET (RULE 26)**

2/6

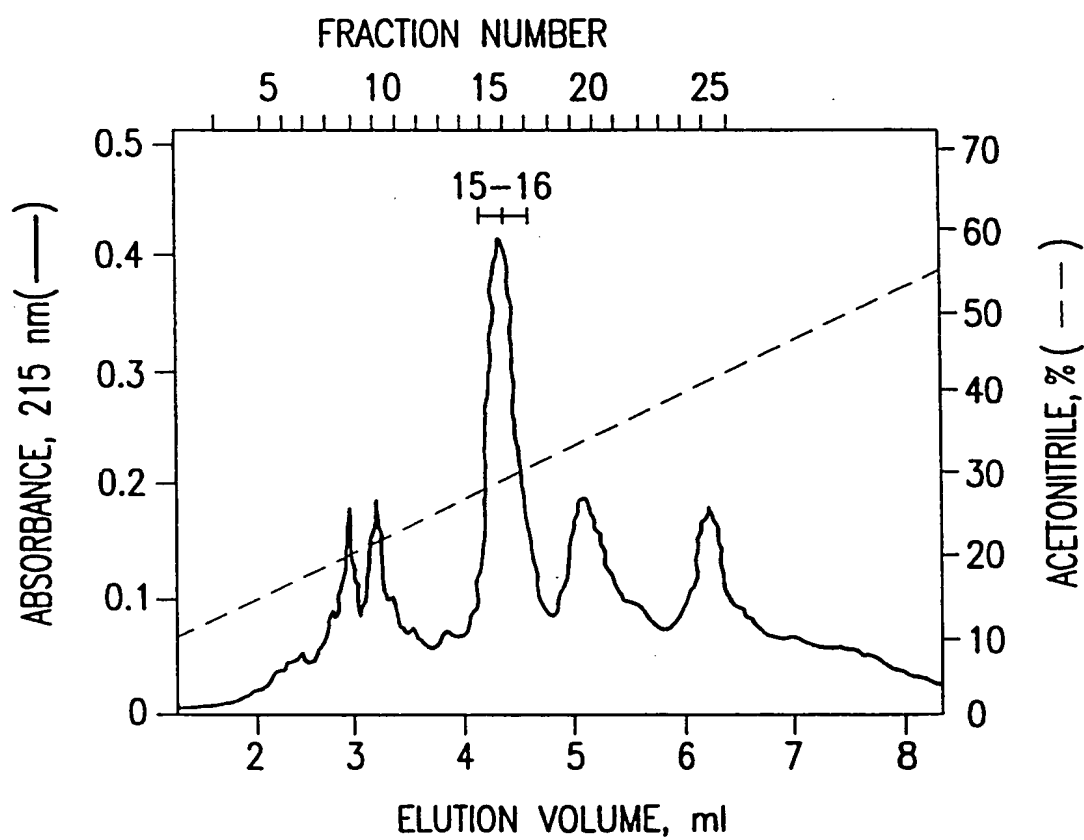


FIG.3

SUBSTITUTE SHEET (RULE 26)

3/6

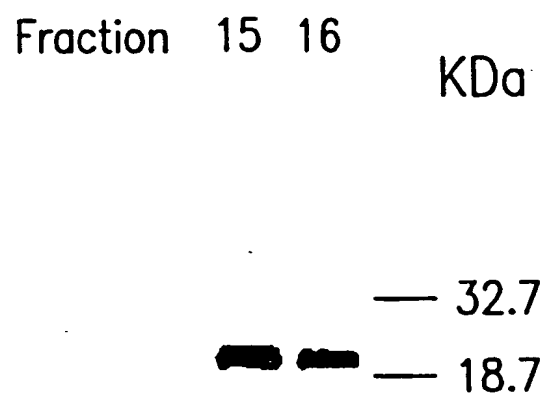


FIG.4

SUBSTITUTE SHEET (RULE 26)

4/6

Fraction 15

KDa

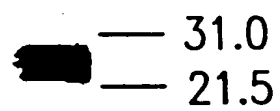


FIG.5

5/6

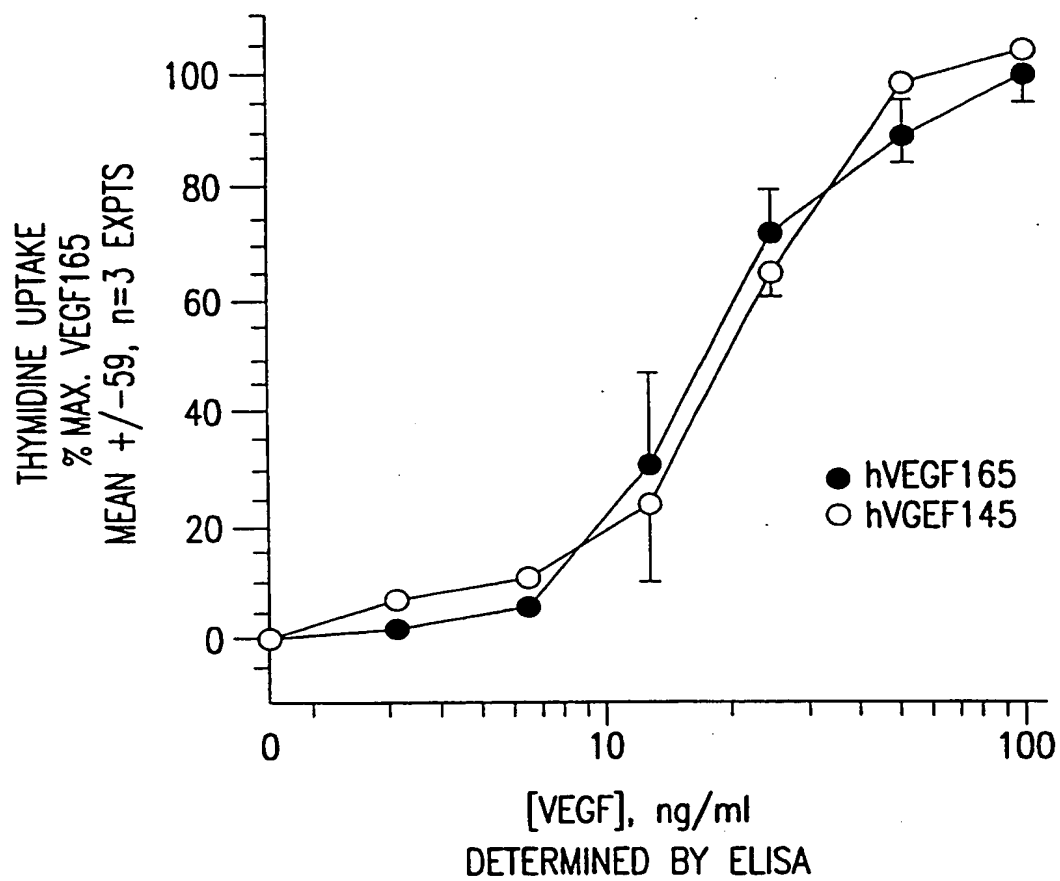


FIG.6

6/6

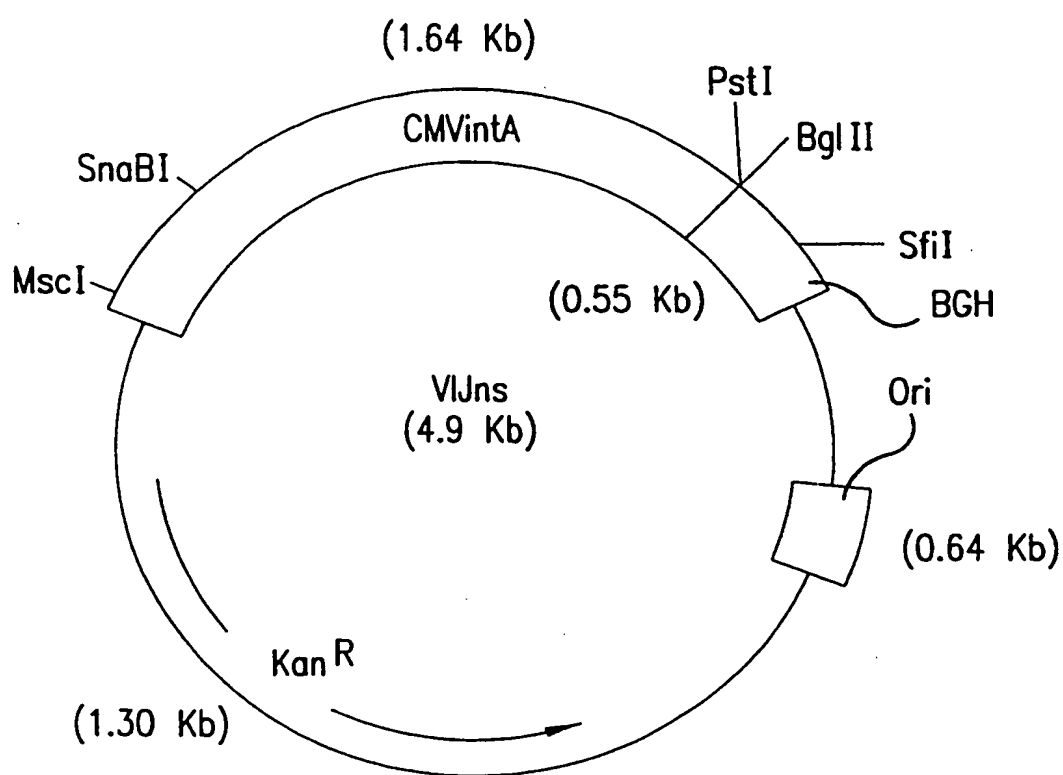


FIG.7

SUBSTITUTE SHEET (RULE 26)



## SEQUENCE LISTING

&lt;110&gt; Merck &amp; Co., Inc.

<120> GENE THERAPY FOR STIMULATION OF  
ANGIOGENESIS

&lt;130&gt; 20073 PCT

&lt;150&gt; 60/063,629

&lt;151&gt; 1997-10-27

&lt;150&gt; GB 9724906.4

&lt;151&gt; 1997-11-26

&lt;160&gt; 5

&lt;170&gt; FastSEQ for Windows Version 3.0

&lt;210&gt; 1

&lt;211&gt; 516

&lt;212&gt; DNA

&lt;213&gt; Human

&lt;400&gt; 1

atgaactttc	tgctctcttg	ggtgcattgg	agccttgcc	tgctgctcta	cctccaccat	60
gccaagtgg	cccaggctgc	acccatggca	gaaggaggag	ggcagaatca	tcacgaagt	120
gtgaagttca	tggatgtcta	tcagcgcagc	tactgccatc	caatcgagac	cctggtggac	180
atcttccagg	agtaccctga	tgagatcgag	tacatcttca	agccatcctg	tgtgccctg	240
atgcatgacg	ggggctgctg	caatgacgag	ggcctggagt	gtgtgcccac	tgaggagtcc	300
aacatcacca	tgcagattat	gcggatcaaa	cctcaccaag	gccagcacat	aggagagatg	360
agcttcctac	agcacaacaa	atgtgaatgc	agaccaaaga	aagatagagc	aagacaagaa	420
aaaaaatcag	ttcgaggaaa	gggaaagggg	caaaaacgaa	agcgcaagaa	atcccgggtat	480
aagtccctgga	gcgtgtgtga	caagccaagg	cggtga			516

&lt;210&gt; 2

&lt;211&gt; 171

&lt;212&gt; PRT

&lt;213&gt; Human

&lt;400&gt; 2

Met	Asn	Phe	Leu	Leu	Ser	Trp	Val	His	Trp	Ser	Leu	Ala	Leu	Leu	Leu
1				5					10					15	
Tyr	Leu	His	His	Ala	Lys	Trp	Ser	Gln	Ala	Ala	Pro	Met	Ala	Glu	Gly
			20					25					30		
Gly	Gly	Gln	Asn	His	His	Glu	Val	Lys	Phe	Met	Asp	Val	Tyr	Gln	
		35					40				45				
Arg	Ser	Tyr	Cys	His	Pro	Ile	Glu	Thr	Leu	Val	Asp	Ile	Phe	Gln	Glu
		50				55					60				
Tyr	Pro	Asp	Glu	Ile	Glu	Tyr	Ile	Phe	Lys	Pro	Ser	Cys	Val	Pro	Leu
65					70				75					80	
Met	Arg	Cys	Gly	Gly	Cys	Cys	Asn	Asp	Glu	Gly	Leu	Glu	Cys	Val	Pro
			85					90					95		
Thr	Glu	Glu	Ser	Asn	Ile	Thr	Met	Gln	Ile	Met	Arg	Ile	Lys	Pro	His
			100					105					110		

Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys  
 115 120 125  
 Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Lys Lys Ser Val  
 130 135 140  
 Arg Gly Lys Gly Lys Gly Gln Lys Arg Lys Arg Lys Lys Ser Arg Tyr  
 145 150 155 160  
 Lys Ser Trp Ser Val Cys Asp Lys Pro Arg Arg  
 165 170

<210> 3  
 <211> 33  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> oligonucleotide

<400> 3  
 acgggatcca aatatgaact ttctgctctc ttg 33

<210> 4  
 <211> 27  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> oligonucleotide

<400> 4  
 tggaagcttt caccgccttg gcttgtc 27

<210> 5  
 <211> 4864  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> E. coli

<400> 5  
 tcgcgcggtt cggatgatgac ggtgaaaacc tctgacacat gcagctcccg gagacgggtca 60  
 cagcttgtct gtaagcggat gccgggagca gacaagcccg tcagggcgcg tcagcgggtg 120  
 ttggcgggtg tcggggctgg cttaactatg cggcatcaga gcagattgta ctgagagtgc 180  
 accatatgcg gtgtgaaata ccgcacagat gcgtaaggag aaaataccgc atcagattgg 240  
 ctattggcca ttgcatacgt tgtatccata tcataatatg tacatttata ttggctcatg 300  
 tccaacatta ccgccatggt gacattgatt attgactagt tattaatagt aatcaattac 360  
 ggggtcatta gttcatagcc catatatgga gttccgcgtt acataactta cggtaaatgg 420  
 cccgcctggc tgaccgcca acgaccccg ccattgacg tcaataatga cgtatgttcc 480  
 catagtaacg ccaataggga ctttccattg acgtcaatgg gtggagtatt tacggtaaac 540  
 tgcccacttg gcagtacatc aagtgtatca tatgccaaagt acgcccccta ttgacgtcaa 600  
 tgacggtaaa tggcccgctt ggcattatgc ccagtacatg accttatggg actttcctac 660  
 ttggcagtag atctacgtat tagtcatcgc tattaccatg gtgatgcggt ttggcagta 720  
 catcaatggg cgtggatagc ggtttgactc acggggattt ccaagtctcc accccaattga 780  
 cgtcaatggg agtttgtttt ggcacaaaaa tcaacgggac tttccaaaat gtcgtaacaa 840  
 ctccgcccc a ttgacgcaa tgggcggtag gcgtgtacgg tgggaggtct atataagcag 900  
 agctcgttta gtgaaccgtc agatcgcctg gagacgccat ccacgctggt ttgacctcca 960

tagaagacac	cgggaccgat	ccagcctccg	cggccgggaa	cggtgcattg	gaacgcggat	1020
tccccgtgcc	aagagtgcg	taagtaccgc	ctatagagtc	tataggccca	cccccttggc	1080
ttcttatgca	tgctatactg	tttttggtt	ggggtctata	cacccccgct	tcctcatgtt	1140
ataggtgatg	gtatagctta	gcctataggt	gtgggttatt	gaccattatt	gaccactccc	1200
ctattggtga	cgatactttc	cattactaat	ccataacatg	gctcttttgc	acaactctct	1260
ttattggcta	tatgccaata	cactgtcctt	cagagactga	cacggactct	gtatttttac	1320
aggatgggg	ctcatttatt	atttacaat	tcacatatac	aacaccaccg	tccccagtgc	1380
ccgcagtttt	tattaaacat	aacgtgggat	ctccacgcga	atctcgggta	cgtgttccgg	1440
acatgggctc	ttctccggta	gcgccgggag	ttctacatcc	gagccctgct	cccatgcctc	1500
cagcgactca	tggtcgctcg	gcagctcctt	gctcctaaca	gtggaggcca	gacttaggca	1560
cagcacgatg	cccaccacca	ccagtgtgcc	gcacaaggcc	gtggcggtag	ggtatgtgtc	1620
tgaatatgag	ctcggggagc	gggcttgac	cgctgacgca	tttgggaagc	ttaaggcagc	1680
ggcagaagaa	gatgcaggca	gctgagttgt	tgtgttctga	taagagtcag	aggttaactcc	1740
cggtgcgggtg	ctgttaacgg	tggaggcgca	tgtagctctga	gcagtactcg	ttgctgcgcg	1800
gcgcgccacc	agacataata	gctgacagac	taacagactg	ttcttttcca	tgggtctttt	1860
ctgcagtcac	cgctccttaga	tctgctgtgc	cttctagtgtg	ccagccatct	gttgttttgc	1920
cctcccccg	gccttccttg	accctggaag	gtgccactcc	cactgtcctt	tcctaataaaa	1980
atgaggaat	tgcatcgcat	tgtctgagta	ggtgtcattc	tattctgggg	ggtgggggtg	2040
ggcagcacag	caagggggag	gattgggaag	acaatagcag	gcatgctggg	gatgcgggtg	2100
gctctatggg	taccaggtg	ctgaagaatt	gaccgggtc	ctcctggggc	agaaagaagc	2160
aggcacatcc	ccttctctgt	gacacaccct	gtccacgccc	ctgggtctta	gttccagccc	2220
cactcatagg	acactcatag	ctcaggagg	ctccgccttc	aatcccaccc	gctaaagtac	2280
ttggagcggt	ctctccctcc	ctcatcagcc	caccaaacca	aacctagcct	ccaagagtgg	2340
gaagaaatta	aagcaagata	ggctattaag	tgcagaggga	gagaaaatgc	ctccaacatg	2400
tgaggaagta	atgagagaaa	tcatagaatt	tcttccgctt	cctcgctcac	tgactcgctg	2460
cgctcggtcg	ttcggtgctg	gcgagcggt	tcagctcact	caaaggcggt	aatacggtta	2520
tcacacgaat	caggggataa	cgagggaaag	aacatgtgag	caaaaggcca	gcaaaaggcc	2580
aggaaccgta	aaaaggccgc	gttgctggcg	tttttccata	ggctccgccc	ccctgacgag	2640
catcacaaaa	atcgacgctc	aagtcagagg	tggcgaaacc	cgacaggact	ataaagatac	2700
caggcggttt	cccctggaag	ctccctcggt	cgctctcctg	ttccgaccct	gcccgttacc	2760
ggatacctgt	ccgcctttct	cccttcggga	agcgtggcgc	tttctcaatg	ctcacgctgt	2820
aggatatctca	gttcgggtgta	ggtcggtcgc	tccaagctgg	gctgtgtgca	cgaaccccc	2880
ggtcagcccg	accgctgcgc	cttatccggt	aactatcgtc	ttgagtccaa	cccggttaaga	2940
cacgacttat	cgccactggc	agcagccact	ggtaacagga	ttagcagagc	gaggtatgta	3000
ggcgggtgcta	cagagttctt	gaagtgggtg	cctaactacg	gctacactag	aaggacagta	3060
tttggtatct	gcgctctgct	gaagccagtt	accttcggaa	aaagagtgtg	tagctcttga	3120
tccggcaaac	aaaccaccgc	tggttagcggt	ggtttttttg	tttgcaagca	gcagattacg	3180
cgcagaaaaa	aaggatctca	agaagatcct	ttgatctttt	ctacggggtc	tgacgctcag	3240
tggaaacgaaa	actcacgtta	agggattttg	gtcatgagat	tatcaaaaag	gatcttcacc	3300
tagatccttt	taaattaaaa	atgaagtttt	aatcaatct	aaagtatata	tgagtaaact	3360
tgggtctgaca	gttaccaatg	cttaatcagt	gaggcaccta	tctcagcgat	ctgtctattt	3420
cgttcatcca	tagttgcctg	actccggggg	gggggggccc	tgaggtctgc	ctcgtgaaga	3480
aggtgtttgt	gactcatacc	aggcctgaat	cgccccatca	tccagccaga	aagtgaggga	3540
gccacgggtg	atgagagctt	tgttgtaggt	ggaccagttg	gtgattttga	acttttgcct	3600
tgccacggaa	cgggtctgcgt	tgtcgggaag	atgctgtgat	tgatccttca	actcagcaaa	3660
agttcgattt	attcaacaaa	gccgcctgcc	cgtcaagtca	gcgtaatgct	ctgccagtg	3720
tacaaccaat	taaccaatcc	tgattagaaa	aactcatcga	gcatcaaatg	aaactgcaat	3780
ttattcatat	caggattatc	aataccatat	ttttgaaaaa	gccgtttctg	taatgaagga	3840
gaaaactcac	cgaggcagtt	ccataggatg	gcaagatcct	ggtatcggtc	tgcgattccg	3900
actcgtccaa	catcaatata	acctattaat	ttccctcgt	caaaaataag	gttatcaagt	3960
gagaaatcac	catgagtgc	gactgaatcc	ggtgagaatg	gcaaaagctt	atgcatttct	4020
ttccagactt	gttcaacagg	ccagccatta	cgctcgtcat	caaaatcact	cgcatacaac	4080
aaaccggttat	tcattcgtga	ttgcgcctga	gcgagacgaa	atacgcgatc	ctgtgttaaaa	4140
ggacaattac	aaacaggaat	cgaatgcaac	cggcgcagga	acactgccag	cgcatacaac	4200
atattttcac	ctgaatcagg	atattcttct	aatacctgga	atgctgtttt	cccggggatc	4260
gcagtgggtga	gtaacctatgc	atcatcagga	gtacggataa	aatgcttgat	ggtcgggaaga	4320

ggcataaaatt	ccgtcagcca	gtttagtctg	accatctcat	ctgtaacatc	attggcaacg	4380
ctacctttgc	catgtttcag	aaacaactct	ggcgcatcgg	gcttcccata	caatcgatag	4440
attgtcgcac	ctgattgccc	gacattatcg	cgagcccatt	tatacccata	taaatcagca	4500
tccatgttgg	aatttaatcg	cggcctcgag	caagacgttt	cccgttgaat	atgggtcata	4560
acaccccttg	tattactggt	tatgtaagca	gacagtttta	ttgttcatga	tgatatattt	4620
ttatcttgtg	caatgtaaca	tcagagattt	tgagacacaa	cgtggctttc	ccccccccc	4680
cattattgaa	gcatttatca	gggttattgt	ctcatgagcg	gatacatatt	tgaatgtatt	4740
tagaaaaata	aacaaatagg	ggttccgcgc	acatttcccc	gaaaagtgcc	acctgacgtc	4800
taagaaacca	ttattatcat	gacattaacc	tataaaaata	ggcgtatcac	gaggcccttt	4860
cgtc						4864

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/22668

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 48/00; C12N 5/00, 15/00

US CL :514/44; 435/320.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Chemical Abstracts

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,652,225 A (ISNER) 29 July 1997, see entire patent, especially at col. 12, line 21 to col. 13, line 29; and col. 17, line 57 to col. 18, line 3.	1-21
Y	POLTORAK, Z. et al. VEGF-145, a Secreted Vascular Endothelial Growth Factor Isoform that Binds to Extracellular Matrix. Journal of Biological Chemistry. 14 March 1997, Vol. 272, No. 11, pages 7151-7158, especially pages 7157 and 7158.	1-21
Y	MUHLHAUSER, J. et al. VEGF-165 Expressed by a Replication-Deficient Adenovirus Vector Induces Angiogenesis in Vivo. Circulation Research. 1995, Vol. 77, pages 1077-1086, especially pages 1081, 1082, 1084 and 1085.	1-21

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 27 FEBRUARY 1999	Date of mailing of the international search report 09 MAR 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer <i>D. Lawrence</i> DEBORAH CROUCH, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)\*

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/22668

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MAGOVERN, C.J. et al. Regional Angiogenesis Induced in Nonischemic Tissue by an Adenoviral Vector Expressing Vascular Endothelial Growth Factor. Human Gene Therapy. 20 January 1997, Vol. 8, pages 215-227, see especially pages 218-220.	1-21

Form PCT/ISA/210 (continuation of second sheet)(July 1992)★